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ACYL SECOIRIDOIDS AND ANTIFUNGAL CONSTITUENTS FROM *GENTIANA MACROPHYLLA*

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Key Word Index—*Gentiana macrophylla*; Gentianaceae; secoiridoids; sterols; triterpenes; disaccharide; chromenes; kurarinone; kushenol I; 2-methoxyanofinic acid; 6′-*O*-β-D-glucosylsweroside; macrophyllosides A–D; antifungal activity; structure–activity relationships.

Abstract—LC–UV–mass spectrometry and bioassay co-directed fractionation of an aqueous acetone extract of the roots of *Gentiana macrophylla* gave three new chromene derivatives and two novel and six known secoiridoids, along with kurarinone, kushenol I, β-sitosterol, stigmasterol, daucosterol, β-sitosterol-3-*O*-gentiobioside, α-amyrin, oleanolic acid, isovitexin, gentiobiose and methyl 2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate. The structures of the new products were established from spectral and chemical evidence as 2-methoxyanofinic acid and macrophyllosides A–D. The six known secoiridoids were gentiopicroside, sweroside, 6′-*O*-β-D-glucosylgentiopicroside, 6′-*O*-β-D-glucosylsweroside, trifloroside and rindoside. The new acid (2-methoxyanofinic acid), its methyl ester, kurarinone and kushenol I were shown to be active against the plant pathogenic fungus *Cladosporium cucumerinum*. The methyl ester and kurarinone inhibited also the growth of the human pathogenic yeast *Candida albicans*. Structure–activity relationships were studied. Thus, addition of a methoxyl group to the benzene nucleus of anofinic acid (2,2-dimethyl-2*H*-1-benzopyran-6-carboxylic acid) increased the antifungal activity remarkably whereas glycosylation at the carboxylic moiety was found to remove the activity. Esterification of the new acid induced its activity against *C. albicans*, but decreased its growth inhibition properties against *C. cucumerinum*. Hydroxylation of kurarinone at the 3β-position removed its activity against *C. albicans* and decreased the inhibition of *C. cucumerinum*. In addition, the chemotaxonomic significance of the identified constituents is discussed.

INTRODUCTION

Roots of *Gentiana macrophylla* Pall., a traditional Chinese drug with a long history of use for the treatment of jaundice, hepatitis, constipation, pains and rheumatism [1], have been chemically investigated by several groups [2–6]. Regarding the active principles of *G. macrophylla*, little, except the isolation of some flavonoids and secoiridoids, has been reported to date. Following our previous investigation of novel constituents from *G. rhodentha* collected in southwestern China [7], we have recently reported the characterization of a novel secoiridoid glycoside and antifungal chromenes (anofinic acid and fomannoxin acid) from *G. algida* growing in the northwestern part of China [8]. In continuation of our phytochemical investigation of Chinese *Gentiana* species, an aqueous acetone extract of the roots of *G. macrophylla* was subjected to LC–UV–mass spectrometry and bioassay screening as outlined in earlier communications [9, 10]. These analyses indicated that the crude extract contained antifungal constituents, benzoylated secoiridoids and compounds giving UV absorption bands very similar to those of anofinic acid [8]. To the best of our knowledge, natural products possessing these characteristics have not been reported from this plant. A detailed chemical and biological study of the extract was therefore performed.

RESULTS AND DISCUSSION

Repeated chromatographic purifications of an aqueous acetone extract of roots of *G. macrophylla* afforded two new (**7** and **8**) and six known (**1**–**6**) secoiridoid glycosides, three novel chromenes (**9**–**11**) and two dihydroflavones, kurarinone (**12**) and kushenol I (**13**), together with two triterpenes (α-amyrin and oleanolic acid), four sterols (β-sitosterol daucosterol, stigmasterol and β-sitosterol-3-*O*-gentiobioside), a flavone C-glycoside (isovitexin), a disaccharide (gentiobiose) and

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a benzoic acid derivative [methyl 2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate].

The aqueous acetone extract was screened by LC–UV–TSP mass spectrometry following our standard chemical screening procedure for the Gentianaceae [9]. The UV and TSP mass spectra of **1–3** recorded on-line (**1**: UV, 270 and 255 nm; TSP mass spectrum, $[M+H]^+$ ion at *m/z* 357) (**2**: UV, 246 nm; TSP mass spectrum, $[M+H]^+$ ion at *m/z* 359) (**3**: UV, 246 nm; TSP mass spectrum, $[M+NH_4]^+$ ion at *m/z* 536) allowed the identification **1–3** as gentiopicroside, sweroside and 6′-*O*-β-D-glucosylgentiopicroside, respectively. The structure of **3** was confirmed by its ^1H and ^{13}C NMR data. The secoiridoids **1** and **2** are both widespread constituents of Gentianaceae [9, 11]; **3** was first characterized from *G. asclepiadea* [12].

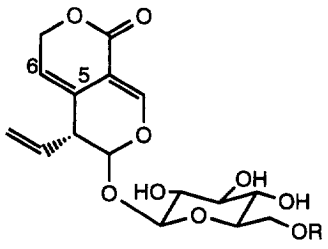
The UV spectrum of **4** was similar to that of **2**, but its molecular weight was 162 amu higher than that of **2** (**4**: UV, 252 nm; TSP mass spectrum, $[M+NH_4]^+$ ion at *m/z* 538). Based on these on-line data, **4** was postulated to be a disaccharide glycoside with an aglycone carbon framework identical to that of **2**. The structure of **3** was confirmed by its ^1H and ^{13}C NMR data to be 6′-*O*-β-D-glucopyranosylsweroside (see Experimental). This secoiridoid has already been characterized from *Swertia punicea* [13] and is known as swertiapunimarin.

Compounds **5** and **6** were shown to be trifloroside and rindoside, respectively, by comparing their UV, TSP mass spectral, ^1H and ^{13}C NMR data with those of the literature [14, 15]. Secoiridoid glycosides **1**, **2**, **5** and **6** were also re-isolated recently from *G. algida* [8].

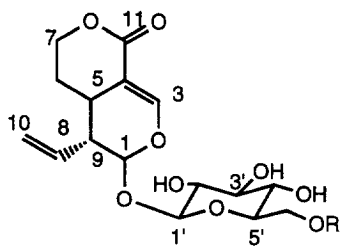
In the positive FAB mass spectrum of **7**, a set of weaker quasimolecular ions were observed at *m/z* 899 $[M+Na]^+$, 877 $[M+H]^+$, together with strong fragment peaks at *m/z* 715 (base peak) and 519 (87%) produced presumably by successive eliminations of glucosyl and dihydroxybenzoyl residues. Therefore, a molecular formula of $C_{40}H_{44}O_{22}$ could be proposed for **7** on the basis of the mass spectral evidence as well as the ^1H and ^{13}C NMR data, including the DEPT experiments. Basic hydrolysis of **7** led to the liberation of **2** and methyl 2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate (see Experimental). In the NMR spectra of **7**, resonances for these two subunits were compared with literature data {**2** [7, 8, 16]; 2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate [14, 17]}. In addition, a 2,3-dihydroxybenzoyl group was revealed by a triplet ($J = 8$ Hz) at δ 6.72 and a pair of double doublets ($J = 8.0$ and 1.4 Hz) at δ 7.22 and 7.03 [8, 18]. Furthermore, the presence of two aromatic residues was clearly indicated by the negative FAB tandem mass spectrometric technique (Fig. 1). These observations, together with a pair of acetoxy singlets at δ 2.09 and 1.88, suggested that **7** was a tetra-acylated secoiridoid bearing two different aromatic moieties. This assumption was confirmed by its ^{13}C NMR spectrum and 2D NMR analyses (^1H–^1H COSY, HETCOR and FLOCK [19]), which led as well to the unambiguous assignment of all proton and carbon resonances (Table 1). In the ^{13}C NMR spectrum of **7**, the chemical shifts of C-1′ to C-6′ were very similar to those of **5**, **6** and gentomoside, a secoiridoid similar to **6**, but with an epoxy function between positions 8 and 10 [14, 15]. These spectral features could only be explained by placing all acyl groups on the saccharide nucleus, i.e. on C-2′, C-3′, C-4′ and C-6′. In the FLOCK spectrum of **7**, the correlation of C-7″ with H-4′ and H-6″ revealed that the 2-hydroxy-3-(1-β-D-glucopyranosyl)benzoyl residue was present at C-4′. Similarly, the 2,3-dihydroxybenzoyl group was shown to be at C-2′ by the long range correlation of C-7‴ with H-2′ and H-6‴. As a result, the two acetoxy groups had to be at C-3′ and C-6′, respectively. Moreover, the low field positions of the protons at C2′-C4′ and C6′ (see Experimental) show that the oxygen atoms at these positions are acylated [14, 15]. In conclusion, the structure of **7** is that shown in the formula and it has been named macrophylloside A.

The positive FAB mass spectrum of **8** gave intense quasimolecular ions at *m/z* 893 $[M+H]^+$ (base peak), 915 $[M+Na]^+$ and 931 $[M+K]^+$. The M_r of **8** (892) was thus 16 amu higher than that of **7** (876). According to the NMR measurements this difference in M_r was due the presence of an additional oxygen atom in **8**. The ^1H and ^{13}C NMR data were similar to those of **7** (Table 1); both had identical UV and IR spectra (see Experimental). The negative FAB tandem mass spectral analysis of **8** also suggested the presence of two aromatic units, identical to those of **7** (Fig. 1). The differences between **7** and **8** were found in the NMR signals of the aglycone secoiridoid moieties. In the ^1H NMR spectrum of **8**, the singlet of H-3 at δ 7.14 (instead of a doublet as in the case of **7**) (see Experimental) and the broadened doublet of H-9 at δ 2.97 coupled with H-1 and H-8 alone suggested that C-5 was quaternary. Further comparisons between the ^1H NMR spectra of **7** and **8** revealed that the multiplet at δ 2.68, assigned to H-5 of **7**, was missing in the ^1H NMR spectrum of **8**, and that the signal of H-7β at δ 4.70 in the case of **8** was shifted downfield by 0.68 ppm from that of **7** at δ 4.02. These findings suggested the presence of a 5β-hydroxyl group in **8**. Comparison with 2′-*O*-acetylswertiamarin [20] showed that the aglycone moiety of **8** was similar to that of swertiamarin. In conclusion, the structure of **8** is that shown in the formula and has been named macrophylloside B.

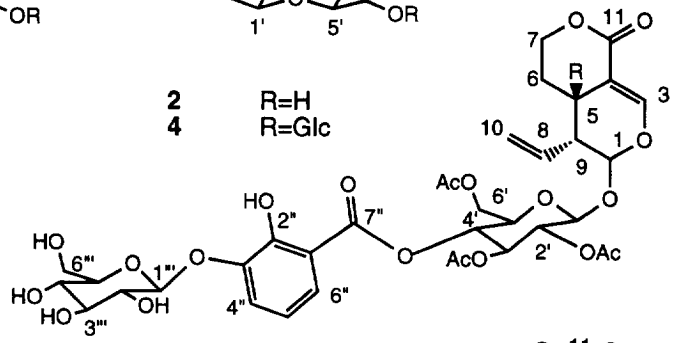
In the TSP mass spectrum of **9**, quasimolecular ions appeared at *m/z* 252 $[M+NH_4]^+$ and 235 $[M+H]^+$. Accordingly, a molecular formula of $C_{13}H_{14}O_4$ could be proposed for **9**, based on the mass data and its ^1H and ^{13}C NMR spectra together with the DEPT experiment (Table 2). In the ^1H NMR spectrum of **9**, the presence of a 2,4,5-trisubstituted benzoyl nucleus was suggested by a pair of singlets at δ 6.50 and 7.59, the latter appearing downfield due to the paramagnetic effect of the carbonyl group. Furthermore, the 2,2-dimethyl-2*H*-chromene moiety was revealed by a six-proton singlet at δ 1.42 and a pair of olefinic doublets ($J = 9.8$ Hz) at δ 6.33 and 5.62, the former showing a homoallylic coupling with the aromatic signal at δ 6.50



1 R=H
3 R=Glc



2 R=H
4 R=Glc



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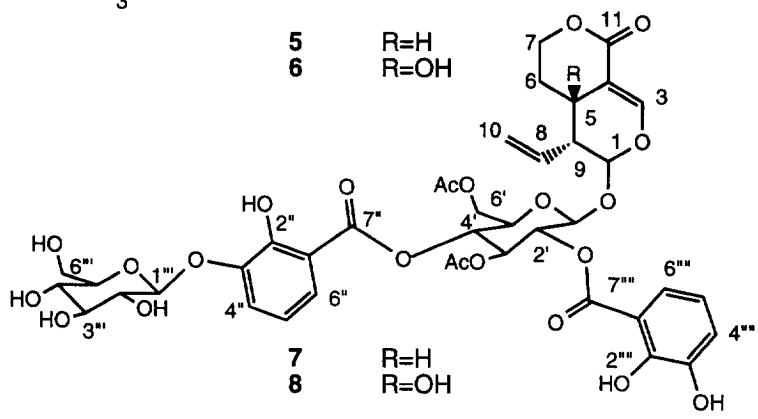
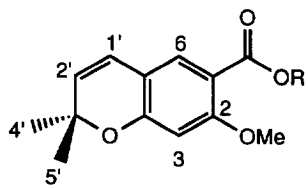
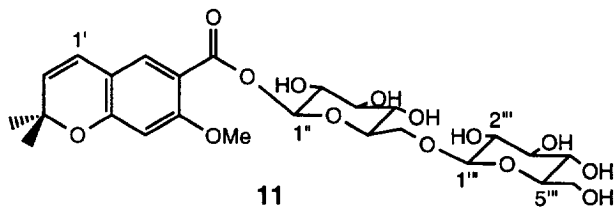
6 R=OH



7 R=H
8 R=OH



9 R=H
9a R=Me



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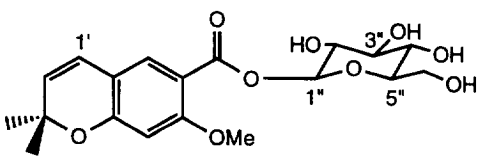

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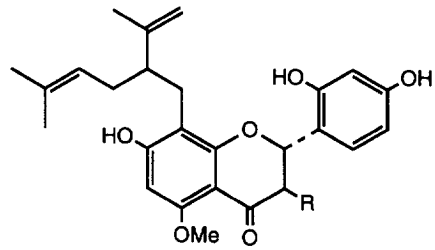

12 R=H
13 R=OH

Table 1. ^{13}C NMR spectral data for compounds **5–8**

C	5	6	7*	8*	DEPT
1	98.6	99.8	97.7	100.1	CH
3	153.3	153.0	153.1	153.3	CH
4	106.8	110.5	106.1	109.5	C
5	28.7	64.2†	28.7	64.2†	CH
6	25.8	33.5	25.6	33.5	CH_2
7	70.0	66.2	69.4	65.8	CH_2
8	132.8	131.1	132.5	133.1	CH
9	43.4	52.1	43.1	51.8	CH
10	121.4	121.6	121.4	121.8	CH_2
11	168.0	167.6	167.0	166.8	C
1′	97.6	98.5	96.7	98.7	CH
2′	73.0	72.4	72.8	72.6	CH
3′	72.3	72.9	72.7	73.1	CH
4′	71.3	70.8	71.2	70.7	CH
5′	73.3	73.1	73.2	73.3	CH
6′	63.3	63.2	63.4	63.2	CH_2
1″	114.5	114.6	114.5	114.5	C
2″	153.4	153.1	152.6	152.6	C
3″	147.4	147.4	147.3	147.5	C
4″	124.9	125.0	124.9	124.9	CH
5″	120.3	120.3	120.4	120.5	CH
6″	124.2	124.3	124.2	124.2	CH
7″	169.4	169.3	169.4	169.3	C
1‴	103.3	103.4	103.2	103.3	CH
2‴	74.8	74.8	74.9	74.8	CH
3‴	77.7	77.7	77.7	77.7	CH
4‴	70.9	71.3	71.0	71.2	CH
5‴	78.3	78.3	78.3	78.3	CH
6‴	62.5	62.5	62.4	62.4	CH_2
1⁗	—	—	112.9	112.7	C
2⁗	—	—	151.2	151.7	C
3⁗	—	—	147.2	147.3	C
4⁗	—	—	121.0	121.0	CH
5⁗	—	—	120.8	120.4	CH
6⁗	—	—	122.7	122.8	CH
7⁗	—	—	170.4	171.1	C
OAc	172.2	172.4	172.2	172.2	C
	171.3	172.2	171.4	171.4	C
	171.0	171.3	—	—	C
	20.6	20.7	20.6	20.6	Me
	20.5	20.5	20.5	20.5	Me
	20.4	20.4	—	—	Me

*Assigned by the ^1H–^{13}C COSY and FLOCK spectra.
†Quaternary carbon signal.

Table 2. ^{13}C NMR spectral data for comounds **9**, **9a**, **10** and **11**

C	9	9a	10	11	DEPT
1	115.3	115.0	114.9	114.9	C
2	162.5	162.8	163.6	163.6	C
3	101.2	101.3	101.3	101.3	CH
4	160.0	159.8	160.5	160.4	C
5	112.2	112.3	111.3	111.2	C
6	131.7*	131.2*	131.7*	131.7*	CH
7	168.8	167.8	165.2	165.1	C
1′	129.9*	129.8*	129.9*	129.8*	CH
2′	122.0	122.0	121.9	121.9	CH
3′	78.8	78.7	78.9	78.9	C
4′,5′	28.6	28.6	28.7	28.7	Me
OMe	56.6	56.4	56.5	56.5	Me
CO_2Me	—	52.1	—	—	Me
1″	—	—	95.7	95.6	CH
2″	—	—	74.0	73.1	CH
3″	—	—	78.8†	77.9†	CH
4″	—	—	71.1	70.9‡	CH
5″	—	—	78.1†	77.7†	CH
6″	—	—	62.3	69.4	CH_2
1‴	—	—	—	104.4	CH
2‴	—	—	—	75.0	CH
3‴	—	—	—	78.0†	CH
4‴	—	—	—	71.4‡	CH
5‴	—	—	—	77.8†	CH
6‴	—	—	—	62.6	CH_2

*,†,‡Signals with the same superscript may be interchanged within the column although the tabulated assignment is most probable.

[21–23]. This deduction was reinforced by the UV absorption bands of **9** (256, 292 and 323 nm), which were very close to those of analogues reported elsewhere [23]. Moreover, a methoxyl group, giving a three-proton singlet at δ 3.88 in the ^1H NMR spectrum of **9**, produced a clear NOE effect (12%) with H-3 and was thus located at the C-2 position. NOE effects were also measured between H-6 and H-1′ (9%) proving that **9** was 2-methoxyanofinic acid.

The NMR spectra of **10** (Table 2) were almost identical to those of **9** except for the presence of a β-glucopyranosyl moiety in the former. The low field H-1″ combined with the high field position of C-1″ proved that this position was acylated [24]. Hence, **10** was the 1-β-D-glucopyranosyl ester of **9**.

Compound **11** was more polar than **10**, but gave the same UV absorption bands as **9** and **10** (see Experimental). In the positive FAB mass spectrum of **11**, an intense molecular ion was exhibited at *m/z* 558, together with a pair of quasimolecular ions at *m/z* 581 $[M + Na]^+$ and 597 $[M + K]^+$ as well as fragment peaks at *m/z* 235 and 217. This observation showed that **11** was most probably a disaccharide glycoside of the new acid **9**. This hypothesis was confirmed by the ^1H and ^{13}C NMR spectral data for **11**, which were similar in part to those of **10** (Table 2). In the ^1H NMR spectrum of **11**, the broadened doublet (J = 11 Hz) at δ 4.17 arising from one of the two protons on C-6″ was shifted downfield when compared to that of **10** (see Experimental). As shown in Table 2, the oxygenated methylene carbon signal of C-6″ (moving downfield to δ 69.4) and a set of signals due to C-1″–C-6″ (assignable to a terminal glucose) indicated that **11** was the gentiobiosyl ester of **9**. We have named **10** and **11** macrophylloside C and D, respectively.

Compounds **12** and **13** gave identical UV absorption bands at 291 and 339 nm, typical of dihydroflavones [25]. The ^1H and ^{13}C NMR data and the specific rotation established firmly the identification of **12** and **13** as kurarinone (first characterized from the roots of *Sophora angustifolia*, Leguminosae [26]) and kushenol

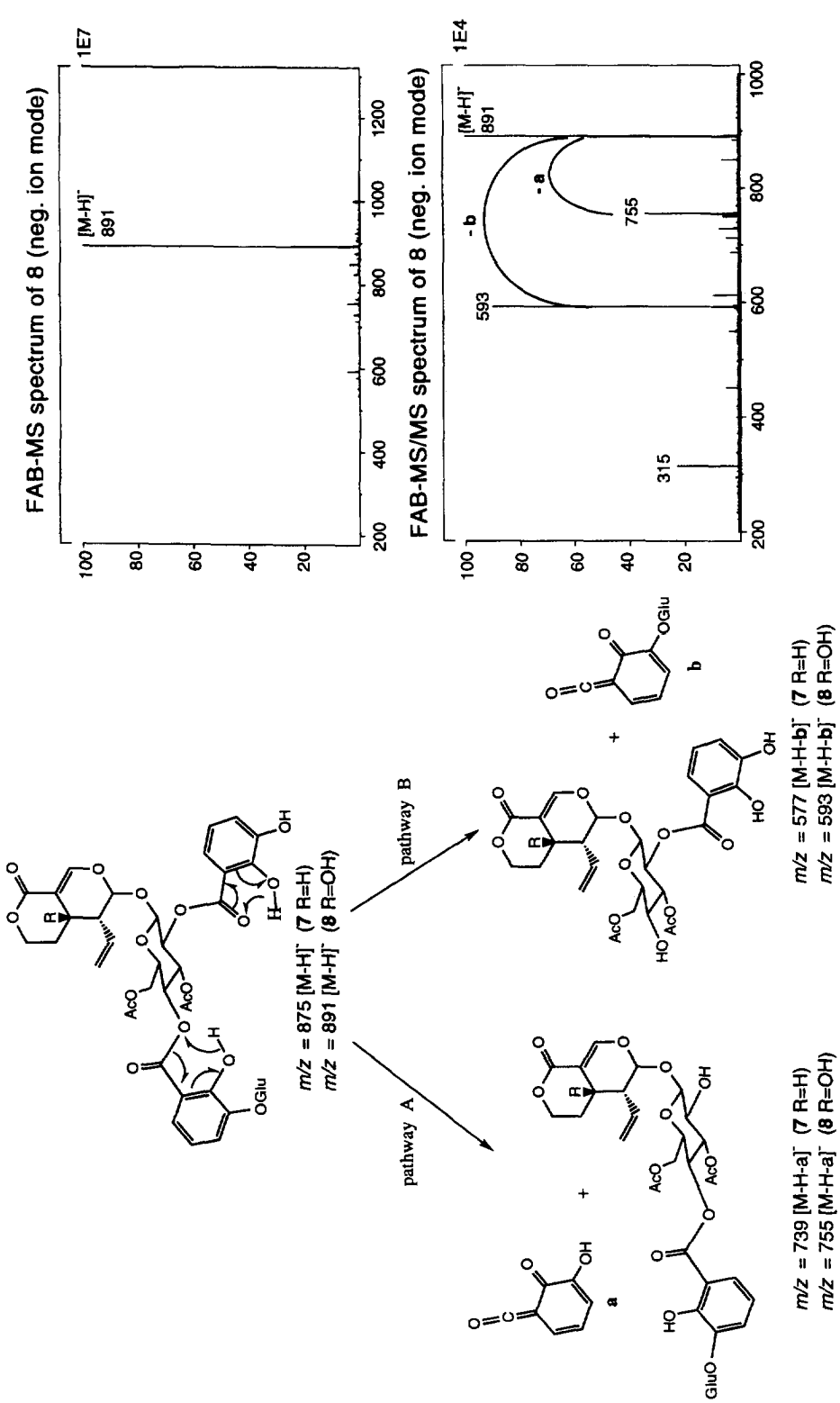

Fig. 1. Competitive negative FAB MS/MS fragmentation pathways of compounds **7** and **8**, indicating the presence of the two different aromatic moieties (for each MS/MS experiment $[M-H]^-$ was chosen as parent ion). The FAB MS and FAB MS/MS spectra of **8** are presented. The FAB MS/MS spectrum was obtained by selecting the $[M-H]^-$ ion at *m/z* 891 as parent ion (CID: argon, 30 ev).

I (previously isolated from *S. flavescens* [27]), respectively.

Following the procedure described previously [10, 28, 29] all identified constituents were subjected to antifungal tests for activities against *Cladosporium cucumerinum* and *Candida albicans*. The new acid **9** and its methyl ester **9a** were found to be active against *C. cucumerinum* at quantities of 0.5 and 10 μg, respectively, in the TLC bioassay. Moreover, the ester **9a** also inhibited the growth of *C. albicans* (quantity required for inhibition: 5 μg). Correlation of their activities with those of anofinic acid (50 μg against *C. cucumerinum*) and the corresponding methyl ester (50 μg against *C. albicans*) [8] demonstrated that introduction of a methoxyl group at the C-2 positions of anofinic acid and its methyl ester increased remarkably their growth inhibition against *C. cucumerinum*. However, the activity was considerably lower than the reference propiconazole (Table 3). In addition, esterification of the acid **9** induced its activity against *C. albicans*. The reference compound miconazole was active in the same test at 0.001 μg. Compounds **10** and **11** (the glycosides of **9**) showed no inhibition against *C. cucumerinum* and *C. albicans* at 200 μg. Compounds **12** and **13** were shown to be active against *C. cucumerinum*, the former inhibiting also the growth of *C. albicans* (Table 3). Previously, **12** was found to be an inhibitor of adenosine 3′,5′-cyclic monophosphate phosphodiesterase [30–32].

The present reinvestigation of *G. macrophylla* (section Aptera) collected in the northwestern part of China shows the close relationships of this species to *G. scabra* var. *buergeri* [15, 33], *G. gelida* [14], *G. asclepiadea* [12], *G. triflora* var. *japonica* [34] (all from the section Pneumonanthe) and *G. algida* (section Frigida) [8, 35]. The sections Aptera, Pneumonanthe and Frigida are known for the absence of xanthones and the presence of various secoiridoid glycosides. Dibenzoylated secoiridoids like **7** and **8** are quite rare as natural products since the only other example known is scabraside, characterized from *G. scabra* (section Pneumonanthe) [33]. The increasing chemical diversity of Gentianaceae was illustrated by the first isolation of 8-lavandulylated dihydroflavones **12** and **13** from the family, as well as by the characterization of the chromene glycosides macrophyllosides C and D.

Table 3. Antifungal activities of compounds **9**, **9a**, **12** and **13**

Compounds	Activity against *C. cucumerinum*	Activity against *C. albicans*
9	0.5*	>200*
9a	19	5
12	5	5
13	50	>200
Miconazole		0.001
Propiconazole	0.1	

*Minimum amount (μg) of compound needed to inhibit fungal growth on TLC plates.

EXPERIMENTAL

TSP-LC-MS analysis and general experimental procedures in this study are the same as described previously [8].

Plant material. Roots of *G. macrophylla* were collected in August 1994 in Pingliang County, Gansu Providence, P. R. China. The material was identified by Associate Prof. L. X. Zhang, with a voucher specimen deposited in the Herbarium of the Department of Biological Sciences and Technology, Nanjing University.

Extraction and isolation. The powdered, air-dried roots (1.1 kg) of *G. macrophylla* were extracted ×2 at room temp. with Me_2CO containing *ca* 2% H_2O. Removal of solvent *in vacuo* from the extract at *ca* 40° gave a brown gum (71 g) which was subsequently dissolved in MeOH. The soln thus obtained was kept overnight at *ca* −10° and then filtered to remove long chain fatty substances pptd upon treatment. Evapn of MeOH from the filtrate afforded a residue (46 g) which was subjected to flash CC (silica gel, 70–230 mesh, 900 g) eluting successively with $CHCl_3$ and a gradient of $CHCl_3$–MeOH (30:1 → 1:9). Based on TLC and HPLC monitoring, 7 frs were collected (F-1: 11 g, F-2: 2.1 g, F-3: 1.5 g, F-4: 2.2 g, F-5: 7.6 g, F-6: 5.1 g and F-7: 3.1 g). CC of F-1 over silica gel (450 g) with petrol (60–90°) containing increasing amounts of Me_2CO gave α-amyrin (156 mg), **9** (17 mg) and a mixt. (288 mg) of sitosterol and stigmasterol (*ca* 2:1). Sepn of F-2 on a silica gel column with a $CHCl_3$–MeOH gradient (50:1 → 1:1) gave sitosterol and a gum which yielded **9** (26 mg) by gel filtration over Sephadex LH-20 with $CHCl_3$–MeOH (1:1). Gel filtration of F-3 with $CHCl_3$–MeOH (1:1) yielded oleanolic acid (86 mg) and a mixt. which gave **12** (8 mg) after semi-prep. HPLC with MeOH–H_2O (9:11). CC of F-4 over silica gel (200 g) with a gradient of $CHCl_3$–MeOH (20:1 → 1:1) afforded f-4/1 and f-4/2. Gel filtration of f-4/1 with $CHCl_3$–MeOH (1:1) gave **12** (7 mg). Gel filtration of f-4/2 with $CHCl_3$–MeOH (1:2) gave pigments, daucosterol and a mixt. which afforded **13** (6 mg) by semi-prep. HPLC with MeOH–H_2O (47:53). CC of F-5 with a gradient of $CHCl_3$–MeOH (20:1 → 1:2) gave five parts (f-5/1, f-5/2, f-5/3, f-5/4 and f-5/5). Gel filtration of f-5/1 with $CHCl_3$–MeOH (1:1) afforded daucosterol (355 mg). Gel filtration of f-5/2 with $CHCl_3$–MeOH (1:2) afforded a mixt. which yielded **5** (35 mg) and **6** (41 mg) after semi-prep. HPLC with MeOH–H_2O (9:11). Gel filtration of f-5/4 in $CHCl_3$–MeOH (2:1) afforded a gum which yielded **7** (43 mg) and **8** (79 mg) by semi-prep. HPLC using MeOH–H_2O (47:53). Gel filtration of f-5/5 in MeOH gave isovitexin (23 mg) and a mixt. which yielded mainly **10** (5 mg) by semi-prep. HPLC with MeCN–H_2O (23:77). CC of F-6 with a gradient of $CHCl_3$–MeOH (10:1 → 1:9) afforded 4 parts (f-6/1, f-6/2, f-6/3 and f-6/4). Gel filtration of f-6/1 with MeOH gave daucosterol (88 mg) and isovitexin (6 mg). Gel filtration of f-6/2 with MeOH afforded mainly **1** (98 mg) and a mixt. of **1** and **2**

(790 mg). Gel filtration of f-6/3 with MeOH–H_2O (9:1) yielded **2** (34 mg) and sitosterol-3-*O*-gentiobioside (14 mg). Gel filtration of f-6/4 with MeOH–H_2O (9:2) gave mainly sitosterol-3-*O*-gentiobioside (23 mg). CC of F-7 over silica gel using a $CHCl_3$–MeOH (9:1 → 1:20) gradient gave 3 frs (f-7/1, f-7/2 and f-7/3). Gel filtration of f-7/1 with MeOH afforded a mixt. which yielded mainly **11** (45 mg) by semi-prep. HPLC with MeOH–H_2O (17:83). Gel filtration of f-7/2 with MeOH afforded methyl-2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate (12 mg) and a mixt. which gave **3** (165 mg) and **4** (28 mg) by semi-prep. HPLC with MeOH–H_2O (13:87). Gel filtration of f-7/3 gave mainly gentiobiose (56 mg).

Bioassays. Bioautography with *C. cucumerinum* and *C. albicans* for evaluating biological activity of the pure products was performed by TLC bioautography [28, 29], and the results are summarized in Table 3.

6′-O-β-D-*Glucopyranosylsweroside* (**4**). Amorphous powder; mp: 133–135°; $[\alpha]_D^{20}$ −15° (MeOH, *c* 0.245); UV λ_{max}^{MeOH} (log ε): 252 (3.8); IR ν_{max}^{KBr} cm^{-1}: 3570–3240 (OH), 1680 (α,β-unsaturated ester); TSP MS *m/z* (rel. int.): 538 $[M + NH_4]^+$, 376 $[M + NH_4 -$ glucosyl$]^+$, 196 $[M + NH_4 -$ gentiobiosyl$]^+$; FAB MS (positive) *m/z* (rel. int.): 521 $[M + H]^+$, 359 $[M + H -$ glucosyl$]^+$; FAB MS (negative) *m/z* (rel. int.) 1039 $[dimer - H]^-$, 619 $[M - H]^-$; ^1H NMR (CD_3OD) δ: 5.51 (1H, *d*, *J* = 1.6 Hz, H-1), 7.59 (1H, *d*, *J* = 4.6 Hz, H-3), 3.11 (1H, *m*, H-5), 1.75 (1H, *m*, H-6a), 1.64 (1H, *m*, H-6b), 4.35 (1H, *m*, H-7a), 3.95 (1H, *m*, H-7b), 5.56 (1H, *ddd*, *J* = 16.0, 10.1, 9.4 Hz, H-8), 2.71 (1H, *ddd*, *J* = 9.4, 5.8, 1.8 Hz, H-9), 5.34 (1H, *dd*, *J* = 16.0, 2.4 Hz, H-10a), 5.27 (1H, *dd*, *J* = 10.1, 2.4 Hz, H-10b), 4.70 (1H, *d*, *J* = 7.8 Hz, H-1′), 4.36 (1H, *d*, *J* = 7.6 Hz, H-1″), 4.18 (1H, *dd*, *J* = 11.6, 1.6 Hz, H-6′a), 3.83 (1H, *br d*, *J* = 11.2 Hz, H-6″a), 3.80–3.20 (10H, *m*, H-2′, H-3′, H-4′, H-5′, H-6′b, H-2″, H-3″, H-4″, H-5″, H-6″b); ^{13}C NMR (CD_3OD, multiplicities by DEPT experiments): δ 98.1 (*d*, C-1),[a] 153.9 (*d*, C-3), 106.0 (*s*, C-4), 28.4 (*d*, C-5), 25.9 (*t*, C-6), 69.8 (*t*, C-7),[b] 133.2 (*d*, C-8), 43.8 (*d*, C-9), 121.1 (*t*, C-10), 168.5 (*s*, C-11), 99.8 (*d*, C-1′),[a] 75.0 (*d*, C-2′),[c] 77.7 (*d*, C-3′),[d] 71.5 (d, C-4′),[e] 78.0 (*d*, C-5′),[d] 69.7 (*t*, C-6′),[b] 104.9 (*d*, C-1″), 74.6 (*d*, C-2″),[c] 77.3 (*d*, C-3″),[d] 71.2 *d*, C-4),[e] 78.0 (*d*, C-5″),[d] 62.7 (*t*, C-6″) ([a–e]interchangeable assignments).

Macrophylloside A (**7**). Amorphous powder, mp: 135–137°; $[\alpha]_D^{20}$ −17° (MeOH; *c* 0.722); UV λ_{max}^{MeOH} (log ε): 237 (4.42), 255 (3.80), 325 (1.21); IR ν_{max}^{KBr} cm^{-1}: 3520–3250 (OH), 1735, 1675 (ester groups), 1605, 1500 (benzene ring); TSP MS *m/z* (rel. int.): 732 $[M + NH_4 -$ glucosyl$]^+$, 596 $[M + NH_4 - \mathbf{a}]^+$, 554 $[M + NH_4 - \mathbf{a} -$ ketene$]^-$, 249, 194, 177; FAB MS (negative) *m/z* (rel. int.): 875 $[M - H]^-$, 739 $[M - H - \mathbf{a}]^-$, 577 $[M - H - \mathbf{b}]^-$; FAB MS (positive) *m/z* (rel. int.): 899 $[M + Na]^+$, 877 $[M + H]^+$, 715 $[M + H -$ glucosyl$]^+$, 579 $[M + H - \mathbf{b}]^+$ (11), 519 $[M + H - \mathbf{b} -$ HOAc$]^+$; ^1H NMR (CD_3OD) δ: 5.40 (1H, *br s*, H-1), 7.27 (1H, *d*, *J* = 1.7 Hz, H-3), 2.68 (1H, *m*, H-5), 1.53 (1H, *m*, H-6a), 1.67 (1H, *br m*, H-6b), 4.02 (1H, *br t*, *J* = 11.1 Hz, H-7a), 4.27 (1H, *m*, H-7b), 5.42 (1H, *m*, H-8), 2.68 (1H, *m*, H-9), 5.31 (1H, *dd*, *J* = 17.1, 1.9, H-10a), 5.26 (1H, *dd*, *J* = 10.0, 1.9 Hz, H-10b), 5.37 (1H, *d*, *J* = 8.1 Hz, H-1′), 5.38 (1H, *dd*, *J* = 8.1, 9.5 Hz, H-2′), 5.80 (1H, *t*, *J* = 9.5 Hz, H-3′), 5.48 (1H, *t*, *J* = 9.5 Hz, H-4′), 4.27 (1H, *m*, H-5′), 4.36 (1H, *dd*, *J* = 12.6, 4.7 Hz, H-6′a), 4.29 (1H, *d*, *J* = 12.6, 2.3 Hz, H-6′b), 7.46 (1H, *dd*, *J* = 8.1, 1.4 Hz, H-4″), 6.88 (1H, *t*, *J* = 8.1 Hz, H-5″), 7.52 (1H, *dd*, *J* = 8.1, 1.4 Hz, H-6″), 4.93 (1H, *d*, *J* = 7.4 Hz, H-1‴), 3.56 (1H, *m*, H-2‴), 3.52 (1H, *m*, H-3‴), 3.44 (1H, *t*, *J* = 9.0 Hz, H-4‴), 3.45 (1H, *m*, H-5‴), 4.06 (1H, *br d*, *J* = 10.9 Hz, H-6‴a), 3.72 (1H, *dd*, *J* = 10.9, 4.4 Hz, H-6‴b), 7.03 (1H, *dd*, *J* = 8.0, 1.4 Hz, H-4⁗), 6.72 (1H, *t*, *J* = 8.0 Hz, H-5⁗), 7.22 (1H, *dd*, *J* = 8.0, 1.4 Hz, H-6⁗), 2.09 (3H, *s*, OAc), 1.88 (3H, *s*, OAc); ^{13}C NMR: Table 1.

Hydrolysis of **7** *with* 0.3 *M NaOH.* A soln of **7** (4.5 mg) in MeOH (0.5 ml) was kept overnight with 0.3 M NaOH (0.2 ml) at room temp. The reaction mixt., after neutralization with HOAc, was concd to a residue which was dissolved in MeOH (0.2 ml). To the soln, an excess of CH_2N_2 in Et_2O was added. The substance obtained was then purified by prep. TLC with $CHCl_3$–MeOH (7:1) to afford sweroside (*ca* 2 mg) and methyl 2-hydroxy-3-(1-β-D-glucopyranosyl)oxybenzoate (*ca* 1.3 mg), both being identified by comparison with authentic samples (co-TLC, co-HPLC and UV spectra).

Macrophylloside B (**8**). Amorphous powder, mp: 127–123°; $[\alpha]_D^{20}$ −9° (MeOH, *c* 0.385); UV λ_{max}^{MeOH} (log ε): 237 (4.42), 256 (3.81), 325 (1.21); IR ν_{max}^{KBr} cm^{-1}: 3520–3230 (OH), 1745, 1680 (ester groups), 1605, 1507 (benzene ring); TSP MS *m/z* (rel. int.): 748 $[M + NH_4 -$ glucosyl$]^+$, 612 $[M + NH_4 - \mathbf{a}]^+$, 476 $[M + NH_4 - 2 \times \mathbf{a}]^+$, 459 $[M + H - 2 \times \mathbf{a}]^+$, 194, 177; FAB MS (negative) *m/z* (rel. int.): 891 $[M - H]^-$, 755 $[M - H - \mathbf{a}]^-$, 593 $[M - H - \mathbf{b}]^-$; FAB MS (positive) *m/z* (rel. int.): 931 $[M + K]^+$, 915 $[M + Na]^+$, 893 $[M + H]^+$, 875 $[M + H - H_2O]^+$ (36); ^1H NMR (CD_3OD) δ: 5.60 (1H, *br s*, H-1), 7.14 (1H, *d*, *J* = 1.7 Hz, H-3), 1.78 (1H, *m*, H-6a), 1.73 (1H, *br d*, *J* = 12.5 Hz, H-6b), 4.70 (1H, *br t*, *J* = 11.0 Hz, H-7a), 4.26 (1H, *m*, H-7b), 5.40 (1H, *m*, H-8), 2.97 (1H, *m*, H-9), 5.32 (1H, *dd*, *J* = 17.0, 1.9, H-10a), 5.28 (1H, *dd*, *J* = 10.2, 1.9 Hz, H-10b), 5.33 (1H, *d*, *J* = 8.1 Hz, H-1′), 5.38 (1H, *dd*, *J* = 8.1, 9.5 Hz, H-2′), 5.82 (1H, *t*, *J* = 9.5 Hz, H-3′), 5.49 (1H, *t*, *J* = 9.5 Hz, H-4′), 4.24 (1H, *m*, H-5′), 4.37 (1H, *dd*, *J* = 12.6, 4.6 Hz, H-6′a), 4.29 (1H, *d*, *J* = 12.6, 1.8 Hz, H-6′b), 7.45 (1H, *dd*, *J* = 8.1, 1.5 Hz, H-4″), 6.88 (1H, *t*, *J* = 8.1 Hz, H-5″), 7.51 (1H, *dd*, *J* = 8.1, 1.5 Hz, H-6″), 4.90 (1H, *d*, *J* = 7.4 Hz, H-1‴), 3.54 (1H, *m*, H-2‴), 3.49 (1H, *m*, H-3‴), 3.43 (1H, *t*, *J* = 9.0 Hz, H-4‴), 3.44 (1H, *m*, H-5‴), 3.91 (1H, *br d*, *J* = 11.1 Hz, H-6‴a), 3.70 (1H, *dd*, *J* = 11.1, 4.6 Hz, H-6‴b), 7.05 (1H, *dd*, *J* = 8.1, 1.5 Hz, H-4⁗), 6.72 (1H, *t*, *J* = 8.1 Hz, H-5⁗), 7.26 (1H, *dd*, *J* = 8.1, 1.5 Hz, H-6⁗), 2.02 (3H, *s*, OAc), 1.86 (3H, *s*, OAc); ^{13}C NMR: Table 1.

2-Methoxyanofinic acid (**9**). Oil; UV λ_{max}^{MeOH} (log ε): 256 (3.98), 292 (0.97), 323 (1.01); IR ν_{max}^{KBr} cm^{-1}: 3490, 1718 (carboxylic group), 1610 (benzene ring);

TSP MS *m/z* (rel. int.): 469 [dimer + H]$^+$, 235]M + H]$^+$; ^{1}H NMR (CD_3OD) δ: 6.50 (1H, *br s*, H-3), 7.59 (1H, *s*, H-6), 6.33 (1H, *br d*, $J = 9.8$ Hz, H-1′), 5.62 (1H, *d*, $J = 9.8$ Hz, H-2′), 1.42 (2H, *s*, H-4′, H-5′), 3.88 (3H, *s*, OMe); ^{13}C NMR: Table 2. Treatment of **9** with CH_2N_2 gave the corresponding ester **9a**: an oil; IR ν_{max}^{KBr} cm^{-1}: 1713 (aromatic ester), 1608 (benzene ring); TSP MS *m/z* (rel. int.): 249 [M + H]$^+$; ^{1}H NMR (CD_3OD) δ: 6.47 (1H, *br s*, H-3), 7.52 (1H, *s*, H-6), 6.17 (1H, *br d*, $J = 9.8$ Hz, H-1′), 5.61 (1H, *d*, $J = 9.8$ Hz, H-2′), 1.42 (2H, *s*, H-4′, H-5′), 3.83 (3H, *s*, OMe), 3.80 (3H, *s*, CO_2Me); ^{13}C NMR: Table 2.

Macrophylloside C (*β-D-glucopyranosyl 2,2-dimethyl-7-methoxy-2H-1-benzopyran-6-carboxylate*) (**10**). Amorphous powder, mp: 72–75°; $[\alpha]_D^{20}$ −23° (MeOH, *c* 0.037); UV λ_{max}^{MeOH} (log ε): 256 (3.98), 292 (0.97), 323 (1.01); IR ν_{max}^{KBr} cm^{-1}: 3500–3200 (OH), 1700 (benzoate carbonyl), 1600, 1475 (benzene ring); TSP MS *m/z* (rel. int.): 235 [M + H]$^+$; FAB MS (positive) *m/z* (rel. int.): 419 [M + Na]$^+$, 397 [M + H]$^+$, 396 [M]$^+$, 381 [M − Me]$^+$, 379 [M − H_2O]$^+$, 235 [M + H − glucosyl]$^+$, 217; ^{1}H NMR (CD_3OD) δ: 6.49 (1H, *br s*, H-3), 7.70 (1H, *s*, H-6), 6.35 (1H, *br d*, $J = 9.8$ Hz, H-1′), 5.63 (1H, *d*, $J = 9.8$ Hz, H-2′), 1.43 (2H, *s*, H-4′, H-5′), 3.84 (3H, *s*, OMe); 5.64 (1H, *d*, $J = 8$ Hz, H-1″), 3.3–3.9 (6H, *m*, H-2″-H-6″); ^{13}C NMR: Table 2.

Macrophylloside D (gentiobiosyl 2,2-dimethyl-7-methoxy-2H-1-*benzopyran*-6-*carboxylate*) (**11**). Amorphous powder; mp: 132–134°; $[\alpha]_D^{20}$ −4° (MeOH, *c* 0.511); UV λ_{max}^{MeOH} (log ε): 256 (3.98), 292 (0.97), 323 (1.01); IR ν_{max}^{KBr} cm^{-1}: 3540–3180 (OH), 1702 (benzoate carbonyl), 1600, 1472 (benzene ring); TSP MS *m/z* (rel. int.): 235 [M + H]$^+$; FAB MS (negative) *m/z* (rel. int.): 557 [M − H]$^-$, 233 [M − H − gentiobiosyl]$^-$; FAB MS (positive) *m/z* (rel. int.): 581 [M + Na]$^+$, 559 [M + H]$^+$, 558 [M]$^+$, 235 [M + H − gentiobiosyl]$^+$, 217; ^{1}H NMR (CD_3OD) δ: 6.48 (1H, *br s*, H-3), 7.69 (1H, *s*, H-6), 6.33 (1H, *br d*, $J = 9.8$ Hz, H-1′), 5.62 (1H, *d*, $J = 9.8$ Hz, H-2′), 1.42 (2H, *s*, H-4′, H-5′), 3.84 (3H, *s*, OMe), 5.62 (1H, *d*, $J = 8$ Hz, H-1″), 4.34 (1H, *d*, $J = 7.6$ Hz, H-1‴), 4.17 (1H, *br d*, $J = 11$ Hz, H-6″b), 3.3–3.9 (11H, *m*, H-2″–H-6″a, H-2‴–H-6‴); ^{13}C NMR: Table 2.

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